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| (54) Title: COMBINATION THERAPY FOR THE TREATMENT OF TUMORS (57) Abstract <p>The present invention relates to a combination therapy method for treating gastrin-dependent tumors. The method comprises the immunization of a patient with an anti-gastrin (17) immunogenic composition in combination with the administration of chemotherapeutic agents such as 5-fluorouracil and leucovorin.</p> | | |

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COMBINATION THERAPY FOR THE TREATMENT OF TUMORS

FIELD OF INVENTION

The invention is related to a tumor therapy for inhibiting growth by neutralizing immunologically the growth stimulating peptide hormones in combination with a chemotherapy
5 applying a 5-fluorouracil derivative and leucovorin.

BACKGROUND OF INVENTION

Gastrin is a peptide hormone which occurs in two mature forms, tetratriacontagastrin (G34) and heptadecagastrin (G17), and is synthesized and secreted by specialized cells, G cells, that are located in the stomach antrum. In gastrin-producing cells, these
10 gastrin hormones are posttranslationally processed from a common precursor molecule termed "preprogastrin" containing a signal peptide. The signal peptide "pre" is removed in the endoplasmic reticulum of the cell, resulting in the "progastrin" peptide, which is in turn further processed in the cell to yield the mature gastrins G34 and G17, before secretion into the bloodstream (Dickinson 1991). (The full citations for the references cited herein are provided in
15 the Reference Section preceding the Claims). Both mature forms of G34 and G17 are amidated at their carboxy-terminal end (-NH₂). In humans, multiple forms of G17 have been found resulting from differential processing of the precursor molecule, each of which may have different biological activities (Dickinson 1995 and Ciccotosto et al. 1995). In the posttranslational processing of gastrin, it is the "mature" carboxy-amidated form that binds to a specific cell
20 receptor, the so-called CCK-B/gastrin receptor, via the carboxy terminus of the peptide (Kopin et al. 1992).

The gastrin hormones are secreted into the circulating blood and bind to specific cells in the stomach, namely, enterochromaffin-like (ECL) cells and parietal cells, that indirectly or directly affect stomach acid output. Historically, both gastrin hormones have been associated
25 with the stimulation of gastric acid secretion (Edkins, J.S. 1905). In recent years, evidence has accumulated showing that gastrin also acts as a trophic factor within the gastrointestinal tract (Johnson, L. 1997) and that it promotes the growth of gastrointestinal cancers (Watson et al. 1989, Dickinson, C.J. 1995), as well as nongastrointestinal cancers, including small cell carcinoma of the lung (Rehfeld et al. 1989).

30 Several types of tumors, including colorectal, stomach, pancreatic and hepatocellular adenocarcinomas possess CCK-B/gastrin receptors in their plasma membranes and the tumor cells respond to gastrin with powerful cellular proliferation (Rehfeld, J.F. 1972, Upp et al. 1989 and Watson et al. 1993). Elevated plasma levels of total gastrin occur in patients with

colorectal cancers, and, in particular, increased amounts of the hormone precursor progastrin have been detected in many colorectal tumors using gastrin antisera (Ciccotosto et al. 1995). More recently, it has been discovered that many of these cancer cells also secrete gastrin and thus effect an autonomous proliferative pathway (Van-Solinge et al. 1993, Nemeth et al. 1993 and Seva et al. 1994).

The peptide hormones G17 and G34 bind to the CCK-B/gastrin receptors on the cell membranes of normal cells. However, it has been found that G17, but not G34, stimulates the growth of gastrin-dependent cancer cells. Serum-associated G17, in particular, has the potential to stimulate the growth of colorectal tumors in an endocrine manner mediated by CCK-B/gastrin receptors in tumor cells (Watson et al. 1993). G17 is particularly implicated in stimulating the growth of colorectal adenocarcinomas due to a possible increased affinity for the CCK-B/gastrin receptors on the tumor cells, as compared to other gastrin hormone species (Rehfeld 1972 and 1993). The CCK-B/gastrin receptors were found to be expressed in a high affinity form on 56.7% of human primary colorectal tumors (Upp et al. 1989).

Numerous studies have shown that, in addition to being able to respond to exogenous endocrine gastrin, human gastric and colorectal tumors produce gastrin and its precursors (Ciccotosto et al., 1995; Finley et al., 1993; Kochman et al., 1992; Nemeth et al., 1993; Van Solinge et al., 1993), thus effecting an autocrine growth stimulatory pathway. Gastrin production in tumor cells differs from that of endocrine G cells. Specifically, those tumor cells contain a high proportion of the precursor progastrin along with a lower concentration of mature peptides. This abnormal ratio is postulated to be due to constitutive unregulated release of gastrin combined with a limited activity of peptidylglycine α -amidating monooxygenase (Ciccotosto et al., 1995; Kelly, 1985). Thus, the unregulated release of gastrin leads to the abnormal production and secretion of different molecular forms of the hormone. Specifically, colon carcinoma cells do not efficiently process progastrin resulting in less conversion of precursor gastrin to the mature peptides and, thus, produce mostly incomplete or aberrant gastrins (Dickinson 1993 and Rehfeld et al. 1993). In addition, the increased gastrin level in colorectal tumors is, in part, attributed to the aberrant expression of the gastrin gene in the colorectal tumor cells (Hoosein et al. 1990, Baldwin et al. 1992 and Finley et al. 1993). Gastrin-like peptides have been identified in such cells (Hoosein et al. 1988, Watson et al. 1991 and Finley et al. 1993), and were confirmed to be precursor gastrin species (Van-Solinge et al. 1993 and Nemeth et al. 1993).

The presence of amidated-G17 (G17-NH₂) in some colorectal cancers (Ciccotosto et al., 1995; Van Solinge et al., 1993) demonstrates that some tumors retain an intact processing

pathway. as gastrin amidation only occurs in secretory granules (Varro et al., 1994).

Endogenously produced gastrin also acts as an autocrine growth factor, since the basal growth of a colorectal cell line was shown to be inhibited by an anti-gastrin antibody (Hoosein et al., 1988). This was confirmed in a second study in which Northern blot analysis revealed gastrin mRNA in the same cell lines and radioimmunoassay revealed gastrin-like immunoreactivity in
5 cell culture supernatant (Hoosein et al., 1990). Gastrin peptides also possess paracrine roles (Watson et al., 1991b) which was confirmed (Finley et al., 1993) in experiments showing gastrin immunoreactivity more predominant in subpopulations of malignant colorectal mucosal cells.

When G17 binds to its receptor a G17/receptor complex is formed which
10 stimulates cell growth by way of secondary messengers for regulating cell function (Ullrich et al. 1990). The binding of G17 to the CCK- B/gastrin receptor leads to activation of phosphatidylinositol breakdown, the protein kinase C activation with a resultant increase in intracellular calcium ion concentration, and the induction of *c-fos* and *c-jun* protooncogenes via the mitogen-activated protein kinase, which has been implicated in the regulation of cell
15 proliferation (Tadisco et al. 1995). Additionally, gastrin binding to the CCK-B/gastrin receptor has been associated with the subsequent increase in phosphorylation by a tyrosine kinase, the pp125FADK(focal adhesion kinase), which may also have a role in the transmission of mitogenic signals (Tanaguchi et al. 1994).

Colorectal cancer remains a formidable disease to treat, as only minor
20 improvements in survival have been obtained in recent years. Surgery is an effective treatment of the primary disease, but it is ineffectual against residual occult disease, which is frequently present. Radiation therapy post-surgery is generally recommended for patients with rectal cancers to reduce the risks of recurrence of the disease. Chemotherapy with 5-fluorouracil(5-FU) has been the most traditional effective therapy following surgery in patients with more advanced
25 colorectal cancers. However, 5-FU therapy has been shown to be only of marginal benefit to the patient, since 5-FU is highly toxic and the therapy is costly and does not appear, alone or in combination with other cytotoxic drugs, to significantly prolong survival. In most instances, occult or inoperable colorectal tumors do not respond well to chemotherapy or radiation, and new treatments are needed to supplement present procedures.

30 Recently, several studies have shown that adjuvant combination chemotherapy with 5-FU and Leucovorin improves the efficacy of 5-FU in patients with advanced colorectal cancer. Leucovorin is a folic acid derivative, also known as folinic acid, Citrovorum factor, or 5-formyl-5,6,7,8,-tetrahydrofolic acid. The studies show that in Dukes' stage C patients, 5-

FU/Leucovorin combination therapy may reduce mortality by 10 to 15% (Moertel, 1994). In the same patient group, combined intravenous and intraperitoneal therapy with 5-FU/leucovorin resulted in a non-significant trend to disease-free survival and overall survival advantage (Scheithauer et al., 1995). In advanced disease, the same drug combination may give rise to a survival advantage (Taylor, 1993), which has been shown to be 13.5 months of median survival in the combination group compared to 7.5 months in 5-FU-treated patients (Petrioli et al., 1995). However, this combination chemotherapy is not without significant morbidity and causes deleterious side effects including stomatitis, diarrhea and myelosuppression (Mahood et al., 1991; Erlichman et al., 1988; Pietnelli et al., 1989), making quality of life an issue, especially in patients with advanced disease.

A number of high affinity CCK-B/gastrin receptor antagonists have been evaluated therapeutically both *in vitro* and *in vivo* in a number of experimental gastrointestinal cancers. For example, proglumide, a glutamic acid derivative (Seva et al. 1990; Harrison et al. 1990 and Watson et al. 1991a); Benzotript, an N-acyl derivative of tryptophan; L-365,260, a derivative of Aspercillin (Bock et al. 1989); and CI-988, a molecule that mimics the C-terminal pentapeptide sequence of CCK (Hughes et al. 1990), have been shown to effectively neutralize the effects of exogenous gastrin on gastrointestinal tumor growth both *in vitro* and *in vivo* (Watson et al. and Romani et al. 1994). However, these antagonists have severe toxic side effects and lack specificity, as they block the action of all potential ligands of the receptor such as G34 and CCK in normal cells. Recently, highly potent and selective CCK-B/gastrin receptor antagonists such as YM022 (Yuki et al., 1997) and YF476 (Takinami et al., 1997) have been also described.

Proglumide and Benzotript have been widely assessed in preclinical studies. The main problem with these compounds is their lack of potency, with relatively high concentrations required to displace G17 (Watson et al., 1992a; Watson et al., 1992b). Despite this, proglumide and Benzotript inhibited the basal and gastrin-stimulated proliferation of a number of cell lines (Seva et al., 1990; Watson et al., 1991a). In addition, proglumide increased the survival of xenograft mice bearing the gastrin-sensitive mouse colon tumor MC26 to 39 days in the treated animals from 25 days in the control animals.

Due to the low specificity of this class of gastrin antagonizing agents for the gastrin/CCK-B receptor, the inhibition of growth is also thought to be induced by a gastrin-receptor-independent action. Moreover, the cellular receptors which recognize and bind the gastrin do not bind all the inhibitors tested (Seva et al. 1994). Thus, if complete inhibition of

gastrin binding to the receptor does not occur in the autocrine growth cascade, the gastrin antagonists may be unable to block this mechanism of tumor growth promotion.

Thus, novel therapeutic approaches are needed both as modalities in their own right and for combination strategies with chemotherapy. Combined treatments offer the possibilities of enhancing the therapeutic index and/or reducing the dose of chemotherapy required, thereby limiting the disadvantageous side effects.

A therapeutic method of selectively immunologically neutralizing the biological activity of the gastrin hormone would provide an effective means of controlling or preventing the pathologic changes resulting from excessive gastrin hormone production associated with colorectal cancers.

Coassigned U.S. Patents Nos. 5,023,077; 5,468,494; 5,607,676; 5,609,870 and 5,622,702 disclose immunogens and immunogenic compositions useful for controlling G17 and G34 levels in a patient by generating anti-gastrin antibodies and also disclose the use of such compositions for the treatment of gastric and duodenal ulcers and gastrin-induced cancers. The present invention concerns the use of the anti-G17 immunogens and immunogenic compositions disclosed in Patent Nos. 5,023,077; 5,468,494; 5,607,676; 5,609,870 and 5,662,702 in a combination therapy with chemotherapeutic agents for treating gastrin-dependent colorectal cancers.

The method of cancer therapy described herein has several advantages over present colorectal cancer treatment methods. The anti-G17 immunization, in combination with chemotherapeutic agents such as 5-FU and Leucovorin, increases the therapeutic effects in controlling or inhibiting colorectal tumor growth over chemotherapy alone.

SUMMARY OF THE INVENTION

The present invention provides a combination therapy for treating tumors comprising immunologically neutralizing peptide hormones and factors which promote tumor cell division in combination with chemotherapy. In particular, the present invention provides a method for treating gastrin-dependent cancers, such as colorectal adenocarcinomas. The method comprises a combination therapy comprising anti-G17 immunization of the patient in need of the therapy, in conjunction with the administration of one or more chemotherapeutic agents. The anti-G17 immunization for treating gastrin-dependent tumors is surprisingly effective in generating anti-G17 antibodies, despite the known myelo-suppressive effects of the chemotherapeutic agents used.

The anti-G17 immunization comprises the active or passive immunization of a patient with an anti-G17 immunogen against the hormone G17 in order to control the patient's G17 levels. As the result of induction of anti-G17 antibodies in a patient, the G17 hormone is neutralized *in vivo* and its physiological effects are inhibited, thereby inhibiting G17-dependent tumor cell growth.

Furthermore, the use of anti-G17 immunization in combination with standard chemotherapy increases the efficacy of colorectal cancer treatment, since in the combination, lower amounts of chemotherapeutic agents may be required to treat a patient, thereby lowering their toxic effects on normal tissues. In addition, the patient's quality of life may be improved and his survival time prolonged.

In a preferred embodiment, the method comprises the active immunization of a mammal possessing a gastrin-dependent tumor with an anti-G17 immunogen, in combination with the administration of one or more chemotherapeutic agents, such as 5-fluorouracil, leucovorin, levamisole, cisplatin, tumor necrosis factor and proglumide. The anti-G17 immunogen may be administered to a patient at the onset of therapy and at subsequent intervals as required by the patient. The anti-G17 antibodies produced by the patient following immunization bind and neutralize G17 in its mature, amidated-G17 as well as its precursor forms, e.g., G17-Gly, *in vivo* and prevent the binding of G17 to its receptors, thereby preventing gastrin-dependent tumor cell growth. The anti-G17 antibody titers produced by an immunized patient may be monitored at predetermined intervals using standard techniques. In addition, the chemotherapeutic agents may be administered as directed by standard regimes or lower doses may be administered as required by the patient.

In another embodiment, the invention further provides a method of treating a gastrin-dependent tumor comprising the passive immunization of a patient possessing a gastrin-dependent tumor with anti-G17 antibodies in combination with one or more chemotherapeutic agents, such as 5-fluorouracil leucovorin, levamisole, cisplatin, tumor necrosis factor and proglumide. In a preferred embodiment of this aspect of the invention, the antibodies may be chimeric, humanized, or human monoclonal antibodies which may be produced by methods well known in the art. The antibodies may be administered together with the chemotherapeutic agents at the onset of therapy and at subsequent intervals after the initial therapy, as required by the patient.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 depicts a graph showing a time scale of serum antibody titers after immunization of rats immunized with 500 µg/ml of rat anti-G17 (1-9)-DT immunogen.

FIG. 2 depicts a graph showing the effects of 30 mg/kg dose of 5-FU/leucovorin treatment on the anti-G17(1-9) antibody titers obtained in rats immunized with the immunogen of the invention.

FIG. 3 depicts a Scatchard plot showing the effects of treatment cycles of 30 mg/kg of 5-FU/leucovorin on the mean white blood cell counts in BDIX rats.

FIG. 4 depicts a bar graph showing the median tumor weight of untreated, anti-G17(1-9) DT-treated and DT-treated rats.

FIG. 5 depicts a bar graph showing the median tumor weights of rats treated with 30mg/kg of 5-FU/leucovorin; 30 mg/kg of 5-FU/leucovorin and DT immunogen; 30 mg/kg of 5-FU/leucovorin and anti-G17(1-9)-DT; 25 mg/kg of 5-FU/leucovorin and DT immunogen; 25 mg/kg of 5-FU/leucovorin and anti-G17(1-9)DT; 20 mg/kg of 5-FU/leucovorin and DT immunogen; 20 mg/kg of 5-FU/leucovorin and anti-G17(1-9)DT; 12.5 mg/kg of 5-FU/leucovorin and DT immunogen; and 12.5 mg/kg of 5-FU/leucovorin and anti-G17(1-9)DT.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides methods of treating tumors, in particular those associated with gastrin-dependent colorectal cancer, with a combination therapy comprising immunizing a patient with an anti-G17 immunogen and treating the patient with chemotherapeutic agents, such as 5-FU and leucovorin. The anti-G17 immunization/5-FU-leucovorin combination therapy, surprisingly, has been found to be more effective than previous therapies in treating colorectal cancer. The chemotherapeutic agents useful in the combination therapy do not significantly inhibit anti-G17 antibody production in an immunized patient and lower doses of chemotherapeutic agents can be used for treating the tumor growth. In addition, the anti-G17 antibody titers produced by immunization are effective to neutralize all forms of G17 hormone.

In a preferred embodiment, the method comprises actively immunizing a patient afflicted with a gastrin-dependent colorectal cancer applying an anti-G17 immunogenic composition in conjunction with administering to the patient chemotherapeutic agents. Subsequent booster anti-G17 immunizations may be administered as required by the patient, as determined by analysis of the patient's serum anti-G17 antibody titers post-immunization, using

standard techniques and standard radiological assessments of the tumors. Anti-G17 immunization may also be provided to a patient prior to tumor surgery.

The anti-G17 immunogens comprise a natural or synthetic peptide fragment of the N-terminal amino acids of G17 as the immunomimic portion of the immunogen. This peptide
5 fragment is conjugated to an immunogenic carrier such as Diphtheria toxoid (DT). In a preferred embodiment of this aspect of the invention, the anti-G17 immunogen comprises the amino-terminal amino acids of G17 from positions 1 through 9, having the amino acid sequence pyroGlu-Gly-Pro-Trp-Leu-Glu-Glu-Glu-Glu, conjugated to Diphtheria toxoid. Other suitable immunogenic protein carriers, include bovine serum albumin, keylimpet hemocyanin, hemocyanin and tetanus toxoid.
10

The immunogens of the invention may also comprise an extension or a spacer peptide sequence suitable for projecting the immunomimic peptide away from the protein carrier and for enhancing its capacity to bind the lymphocyte receptors. A suitable spacer peptide sequence is the amino acid sequence SSPPPPC (SEQ ID NO.: 2 in the Sequence Listing).
15 However, other spacer peptides would be suitable as well. In a preferred embodiment of this aspect of the invention, the preferred spacer sequence is attached to the carboxy-terminal end of the immunomimic peptide. The immunogens of the invention are produced by standard techniques and are disclosed in U.S. Pat. Nos. 5,023,077; 5,468,494; 5,607,676; 5,609,870; 5,688,506 and 5,662,702, the disclosures of which are hereby incorporated by reference.
20 Following immunization, the immunogens of the invention produce high affinity, neutralizing antibodies for inhibiting the effects of G17 in its mature and precursor forms on tumor growth in immunized animals. The anti-G17 antibodies produced bind and neutralize mature and precursor G17, thereby preventing the binding of G17 to the receptors on tumor cells and ultimately inhibiting tumor cell growth. The immunogens raise antibodies which neutralize both the
25 carboxy-amidated and glycine-extended G17, and show no cross-reactivity with G34 or CCK.

The compositions in which the immunogens for active immunization are administered for the treatment of gastrin-dependent tumors in patients may be in a variety of forms. These include, for example, solid, semi-solid and liquid dosage forms, such as powders, liquid solutions, suspensions, suppositories, and injectable and infusible solutions. The preferred
30 form depends on the intended mode of administration and therapeutic applications. The compositions comprise the present immunogens and suitable pharmaceutically acceptable components, and may include other medicinal agents, carriers, adjuvants excipients, etc., which can be mixed using standard procedures. Preferably, the compositions are in the form of unit

doses. The amount of active compound administered for immunization or as a medicament at one time, or over a period of time, will depend on the subject being treated, the manner and form of administration, and the judgment of the treating physician.

5 An effective dosage ranging from 0.001 to 2 mg of the immunogenic composition is administered to the patient for the treatment of the gastrointestinal cancer. The effective dosage of the immunogenic composition is capable of eliciting an immune response in a patient of effective levels of antibody titer to bind and neutralize mature and precursor G17 for 1-3 months after immunization. Following the immunization and the chemotherapeutic agent treatment, with, for example, 5-FU/Leucovorin, of a patient with colorectal cancer, the effectiveness of the
10 therapy on tumor growth is assayed by standard clinical procedures, such as ultrasound and magnetic resonance imaging (MRI) to detect the presence and size of tumors, if any. The anti-G17 antibody titers may also be monitored from a sample of blood taken from the patient.

Booster immunizations should be given as required to maintain an effective antibody titer. Effective treatment of gastrin-dependent colorectal adenocarcinoma and other
15 gastrin-dependent cancers such as stomach, liver, pancreatic and small cell carcinoma of the lungs according to this method should result in inhibition of tumor growth and a decrease in size of the tumor.

For passive immunization, the anti-G17 antibodies are administered to a patient intravenously using a pharmaceutically acceptable carrier, such as saline solution, for example,
20 phosphate-buffered saline.

The chemotherapeutic agents are administered at doses recommended in standard regimes and may be administered at the onset of therapy simultaneously with anti-G17 immunogen, prior to immunization or after immunization. In some cases, it may be beneficial to administer the chemotherapeutic agent both before and after immunization. Subsequent
25 chemotherapeutic treatments may also be administered as required by the patient following evaluation by MRI and ultrasound imaging.

The following experiments were conducted to demonstrate the effects of the present combination therapy on colorectal cancers.

EXAMPLE 1

30 The following experiments were conducted to determine the potential clinical benefit offered by anti-G17(1-9)-DT. The aims of this study were as follows:

(a) to determine the long term effect of specific rat anti-G17(1-9)-DT immunization on the histological appearance of the rat GI tract.

(b) to evaluate the effect of 5-FU/Leucovorin combinations on antibody titers raised by anti-G17(1-9)-DT; and

(c) to determine the therapeutic effect of anti-G17(1-9)-DT and 5-FU/leucovorin combinations on a rat colon model.

5 *Cell line*

DHDK12 is a rat colonic epithelial tumor cell line (Martin, 1983). The cell line was maintained in RPMI 1640 growth medium (Gibco, Paisley, Scotland) containing 10% fetal calf serum (FCS, Sigma, Poole, UK) in humidified conditions at 37°C and 5% CO₂.

Immunogen

10 The anti-G17(1-9)-DT immunogen consists of amino acid residues 1-9 of G17 linked via the carboxy-terminus to the peptide spacer SSPPPPC (SEQ ID NO.: 1 in the Sequence Listing), which in turn is conjugated to DT. The immunogen used in these studies was made specific for rat G17 by replacing the human G17 epitope with the amino terminal 9 amino acids of rat G17, linked through a peptide spacer to diphtheria toxoid (DT). Antiserum raised by rat anti-
15 G17(1-9)-DT was denoted as anti-rat G17 (1-9):DT.

Experimental animals

Male and female BDIX rats were provided by the Cancer Studies Unit, University of Nottingham, UK and were 6-10 weeks old, weighing 340-420 g. The rats were housed in pairs and maintained in a cycle of 12-hour light and 12-hour dark at 25°C with 50% humidity.

20 Prior to each experiment, the animals were grouped to equalize weight distribution. Group sizes ranged from 6-13 animals. The UK Coordinating Committee for Cancer Research (UKCCCR) guidelines were adhered to throughout all animal experimentation.

Immunization procedure

Rat anti-G17(1-9)-DT was dissolved in sterile saline (0.9%), pH 7.3 to 1 mg/ml.
25 The adjuvant, nor-muramyl dipeptide (Peninsula Labs., Belmont, CA, USA), was added to the conjugate solution to give a final conjugate concentration of between 200 and 500 µg/ml. The aqueous solution was formulated with an oily vehicle (montanide ISA 703; AMS Seppic Inc., Paris, France) in a 1:2 ratio (v/v) by emulsification. After placing in a glass syringe which was attached to a second syringe through a three-way stopcock, the mixture was forced back and
30 forth through the syringes 40 times to form an emulsion. An emulsion containing DT peptide and muramyl dipeptide was similarly formulated for control rats. A 200µl volume of emulsion (50 µg/rat) was injected s.c. (right hand flank of the experimental animals). The animals were immunized with either a single injection or repeatedly at 21 day intervals as detailed below.

Cytotoxic treatment regime

Rats received 12.5 and 25 mg/kg of 5-Fluorouracil (5-FU, David Bull Labs., Warwick, UK) and 12.5-25 mg/kg Leucovorin (Lederle Labs., Gosport, Hants, UK) administered intravenously (iv) on days 1, 3 and 5 with the cycle being repeated every 4 weeks over the duration of the study period (Asao, 1992). The cytotoxic combination was administered to the rats either prior to or after anti-G17(1-9)-DT immunization (200 µg/ml).

Initiation of tumor growth

DHDK12 cells were suspended in sterile phosphate buffered line (PBS, Oxoid, Hants., UK) at a cell concentration of 2.5×10^7 /ml. Rats were anesthetized by a 1 ml intraperitoneal injection of Hypnorm (0.315 ng/ml fentanyl citrate and 10 mg/ml fluanisone; Janssen, Berrse, Belgium), Hypnovel (5 ng/ml midazolam; Roche, Basel, Switzerland), and sterile distilled water in a 1:1:5 ratio. Following a subcutaneous (s.c.) incision on the right flank, a 200 µl volume of cell suspension was injected into the muscle layer of the abdominal wall and the surgical incision closed by wound clips. Each experimental group was composed of between 6 and 13 animals.

Determination of specific antibody levels of rat anti-G17(1-9)-DT-immunized rats

To obtain blood samples for analysis, rats were tail-bled at various time points throughout the experiment and at termination by cardiac puncture under terminal anesthesia. Serum anti-rat-G17 antibody levels were determined by enzyme-linked immunosorbent assay (ELISA). A rat G17-bovine serum albumin (BSA) conjugate was dissolved to 2 µg/ml in 0.1M glycine buffer (pH 9.5) and 25 µl per well was plated into 96-well Immunulon U plates (Dynatech Labs., Sussex, UK). The wells were incubated overnight at 4°C after which the unadsorbed conjugate was flicked out and the wells were washed with buffer (0.9% saline, 0.5% Tween-20 [Sigma], 0.02% NaN_3 [Sigma], pH 7.3). This buffer was used for all washing steps and reagent dilutions. Sera were treated at 10-fold serial dilutions, starting at a dilution of 1:100. The positive control was rat anti-rat G17(1-9)-DT antiserum from previously immunized animals and the negative controls were normal rat serum, and serum from rats immunized with DT only. All control sera were used at the same dilutions as the test sera. The diluted sera were added to the wells in 25 µl aliquots in the presence or absence of 25 µl/well rat G17-BSA at 100 µg/ml (as a soluble inhibitor). Baseline control wells received 25 µl assay buffer only. The plates were incubated for 60 minutes at room temperature before washing with the assay buffer. Goat anti-rat immunoglobulin (H+L)-biotin (Zymed, San Francisco, CA, USA) was added to the wells at a 1:500 dilution, 50 µl/well and incubated for 60 minutes in the dark at room temperature. After

washing, avidin-alkaline phosphatase (Zymed), 1:100 dilution was added (50 µl/well) and the plates were incubated for 60 mins. at room temperature. After further washing, p-nitrophenylphosphate (pNPP) substrate (Sigma) was added to the wells at 50 µl/well and after a 5-minute developing time, the absorbance was read at 405 nm. The difference in absorbance between untreated sera and sera co-incubated with rat G17-BSA was calculated as the specific absorbance.

Determination of white blood cell counts

Heparinized blood from the rats was collected by tail bleeds during the experiment and by cardiac puncture at the termination of the experiment. The numbers of white blood cells were analyzed by the Hematology Department at the University Hospital, Nottingham with the use of a FACScan.

Histology

At termination of the long-term anti-G17(1-9)-DT-immunized rats, representative areas of the stomach, colon and rectum from the immunized rats and age-matched controls were dissected and formalin-fixed. The sections were then embedded in paraffin and 4 µm sections were cut by use of a microtome. These were stained by hematoxylin and eosin and evaluated by a histopathologist who had no knowledge of the treatment groups.

Crypt cell proliferation rate

One hour prior to animal termination, vincristine (2 mg/kg, Sigma) was injected intraperitoneally to induce metaphase arrest in the colonic epithelium prior to the assessment of colonic crypt cell proliferation (CCPR). The number of cells in metaphase per crypt were counted. The colon and rectum were removed from each rat, opened longitudinally and mucosa from each fixed in Carnoy's solution. Crypts were gently squashed, longitudinally, under a dissecting microscope and the number of cells in metaphase enumerated (magnification x 25).

Statistical analysis

In vivo results were analyzed by a Mann Whitney non-parametric test by use of the SPSS statistical package for the IBM PC.

Long term anti-G17(1-9)-DT studies

Five male rats were immunized with rat anti-G17(1-9)-DT immunogen as described above, and their antibody titers were measured for a period of 34 weeks following a single immunization. At this point, the rats were boosted with a second injection of rat anti-G17(1-9)-DT. The results are shown in FIG. 1. FIG. 1 shows the time-scale up to 40 weeks after immunization of antibody

titers from rats immunized with 500 µg/ml of rat anti-G17(1-9)-DT. Each point represents an individual animal. Antibody titers were measured by an ELISA assay as described above using a 1:100 dilution of sera. Immunizations are indicated by the arrow. Following the primary immunization, 4 of the 5 rats responded to the rat anti-G17(1-9)-DT immunogen. Antibodies, following this single injection, were detectable by week 7 in 3 of 5 rats and in 4 of 5 rats by week 9. This initial surge of antibodies was followed by a second surge between 15-20 weeks, after which the antibody titers steadily declined and were approaching zero by week 34. At this point, FIG. 1 also shows that after a second immunization with rat anti-G17(1-9)-DT, all rats had detectable anti-rat-G-17 antibody titers within 1-2 weeks post-immunization.

EXAMPLE 2

Histological analysis of the long term anti-G17(1-9)-DT-immunized rats

Specimens from the stomach, colon and rectum were evaluated histologically following hematoxylin and eosin staining as described in EXAMPLE 1. These were compared to specimens from age and sex-matched control rats. All areas of the GI tract evaluated were identical in both anti-G17(1-9)-DT-treated and age-matched control rats with respect to length of villae/crypts/mucosal height. In the stomach, enterochromaffin-like (ECL) cells were similar in number and appearance in the two subject animal groups. However, there was some evidence of granulation of the G cells in the anti-G17(1-9)-DT-treated rat stomach mucosa.

EXAMPLE 3

Crypt cell proliferation rate (CCPR) of colonic epithelium from long term anti-G17(1-9)-DT-immunized rats

The CCPR of colonic epithelium and anti-rat G17-antibody titers were analyzed as described above. Table I shows the results obtained from 4 of 5 rats evaluated comparing CCPR to anti-rat G17 antibody titers. The mean CCPR for control rats was 18.93 (standard deviation 3.2) and for the anti-G17(1-9)-DT-immunized rats 23.7 (standard deviation 7.9). There was no statistical difference in CCPR between the anti-G17(1-9)-DT-immunized and age-matched control rats. These results indicate that the rate of crypt cell division in a colonic epithelium is the same for control and anti-G17(1-9) DT-immunized rats.

Table I A comparison of anti-rat G17:DT antibody titers with the crypt cell proliferation of the colon

| Rat | Specific absorbance relating to anti-rat G17:DT antibodies (1:1000 dilution) | Crypt cell proliferation rate (mean metaphases/crypt after 2 hours vincristine treatment) |
|-----------------|---|--|
| Control 1 | 0 | 21.9 |
| Control 2 | 0 | 19.4 |
| Control 3 | 0 | 14.4 |
| Control 4 | 0 | 20.0 |
| Immunized rat 1 | 0.280 | 29.7 |
| Immunized rat 2 | 0.340 | 30.3 |
| Immunized rat 3 | 0.415 | 13.6 |
| Immunized rat 4 | 0.420 | 21.1 |

EXAMPLE 4

Effect of pre- and post-cytotoxic treatment on antibody levels raised by rat anti-G17(1-9)-DT

5 Rats were injected intravenously with a 1:1 ratio of 5-FU/Leucovorin at 30 mg/kg as described in Example 1 prior to or after anti-G17(1-9)-DT immunization. Each group consisted of 6 male and 6 female rats per group and the mean antibody titers were measured by an ELISA technique using a 1:100 dilution of sera. Antibody levels in each rat were measured from blood samples as described in Example 1.

10 FIG. 2 shows the effect of pre- and post-cytotoxic treatment with 30 mg/kg of 5-FU/Leucovorin cycles on antibody titers raised by anti-G17(1-9)-DT immunization(500 µg/ml). In the Figure, the data is represented as follows: —□— no cytotoxics, 7 immunizations; —◆— 2 immunizations prior to 4 cytotoxic treatments; —○— 1 immunization prior to 4 cytotoxic treatments; —Δ— 1 cytotoxic prior to 4 immunizations (2 cytotoxic treatments during immunizations); —▣— 2 cytotoxic treatments prior to 4 immunizations; —*— 3 cytotoxic treatments prior to 3 immunizations; and —●— 4 cytotoxic treatments prior to 2 immunizations.

20 FIG. 2 shows the mean of 6 female and 6 male rats per group. The standard deviations were around 10% of the mean. There was no significant effect on antibody titers by pre-treatment with the cytotoxic 5-FU/Leucovorin combination on either the antibody levels achieved or the time taken to achieve those levels when compared to untreated anti-G17(1-9)-

DT-immunized rats. The maximum number of treatment cycles evaluated was 4 cytotoxic treatment cycles followed by 2 immunizations.

Figure 3 shows the effects of treatment on mean white blood cells (WBC) counts, in BDIX rats.

5 The effect of cytotoxic treatment with 30 mg/kg 5-FU/Leucovorin in BDIX rats receiving 4 cytotoxic treatments prior to 2 immunizations on the mean white blood cell (WBC) counts is shown in FIG. 3. As shown in the Figure, there was a significant reduction in WBC counts in the representative rats evaluated, post cytotoxic treatment ($p < 0.005$, Students' t-test). The counts were reduced by the number of cytotoxic treatment cycles, indicative of some
10 myelosuppression. However, there was no effect on the antibody response to anti-G17(1-9)-DT produced by the rats, as shown in FIG. 2.

EXAMPLE 5

Effect of combination therapy of 5-FU/Leucovorin and anti-G17(1-9)-DT on the in vivo growth of DHDK12 tumors

15 The effects of combined therapies with 5-FU/Leucovorin (12.5-30 mg/kg) and rat anti-G17(1-9)-DT (200 µg/ml) on the growth of the rat colon tumor DHDK12 cell line in the muscle layer of the abdominal wall of BDIX rats were tested by comparison to tumors in control animals as described in the previous Examples. At the end of the therapies the rats were killed, their tumors excised and weighed using standard procedures. Each group consisted of 10-12
20 rats/group of mixed sex. The median tumor weights are shown with the inter-quartile ranges above the columns. Statistical assessment was done by a Mann Whitley U non-parametric test as described in Example 1.

FIGs. 4 and 5 show the effect of anti-G17(1-9)-DT immunization on the median final tumor weights from BDIX rats implanted with DHDK12 tumor cells in the muscle layer of
25 the abdominal wall. This route of implantation results in a well-vascularized tumor amenable to therapies administered into the circulation (Watson, 1996). Rat anti-G17(1-9)-DT had previously been shown to inhibit final DHDK12 tumor weight by 56.5% when administered at a dose of 500 µg/ml (Watson, 1996). In the present experiments, to detect any benefits of combination therapy with 5-FU/Leucovorin, the anti-G17(1-9)-DT dose was dropped to 200 µg/ml, which resulted in
30 a significant inhibition of tumor growth of 25.7% as shown in FIG. 4. FIG. 4 shows data from tumors excised from untreated control rats, anti-G17(1-9)-DT immunized rats and DT-immunized rats. After a 50 day time period untreated rats had a median tumor weight of 4.43 g. DT immunization resulted in a median tumor weight of 4.7 g, which was not significantly different

from the tumor weights of untreated rats, but which was significantly greater than the median tumor weight of anti-G17(1-9)-DT-immunized rats (3.49 g, $p=0.034$, Mann Whitney).

FIG. 5 shows that 5-FU/Leucovorin alone, given at 30 mg/kg, significantly reduced tumor weight to a median of 1.01 g ($p=0.0106$ when compared to untreated control rats).

5 When rats were treated with the same cytotoxic dose of 5-FU/Leucovorin together with DT immunization, the median tumor weight was not significantly different (0.945 g).

A combination of 5-FU/Leucovorin at 30 mg/kg and rat anti-G17(1-9)-DT immunization resulted in a median tumor weight of 0.68 g which was not significantly different from the 5-FU/Leucovorin/DT-treated group ($p=0.27$). The combination of 25 mg/kg 5-
10 FU/Leucovorin and DT immunization resulted in a median tumor weight of 0.96 g compared to a mean tumor weight of 0.68g in the anti-G17(1-9)-DT-immunized in conjunction with 5-FU/Leucovorin combination therapy group which was not significant ($p=0.409$). When the 5-FU/Leucovorin dose was reduced to 20 mg/kg, the 5-FU/Leucovorin/DT immunogen combination resulted in a median tumor weight of 1.23 g. The median tumor weight was
15 significantly reduced to 0.71 g when 20 mg/kg of 5FU/leucovorin was combined with anti-G17(1-9)-DT immunization ($p=0.027$, Mann Whitney).

Finally, FIG. 5 also shows that a 5-FU/Leucovorin dose of 12.5 mg/kg combined with anti-G17(1-9)-DT immunization ($p=0.015$, Mann Whitney) reduces the median tumor weight from 1.34 g to 0.41 g. 5-FU/Leucovorin-anti-G17(1-9)-DT combinations were compared and no
20 statistically significant difference existed between anti-G17(1-9)-DT given in combination with either 12.5, 20 or 30 mg/kg of 5-FU/Leucovorin.

Due to the limited benefit shown for combination chemotherapy with 5-FU/Leucovorin in both an advanced cancer state and, in particular, with an adjuvant therapy treatment setting (Moertel, 1994; Scheithauer, 1995; Taylor, 1993; Petrioli, 1995), new
25 therapeutic modalities may need to be given either in conjunction with 5-FU/Leucovorin to enhance the therapeutic index (and possibly reduce the chemotherapeutic dose to limit toxicity) or as a second line treatment if chemotherapy fails to be effective. Thus new treatments must be amenable for such use. Immunotherapeutic approaches in conjunction with chemotherapy were previously thought to be problematic due to the myelosuppression associated with
30 chemotherapeutic agents, such as that seen with 5-FU/Leucovorin (Mahood, 1991). In the present study, however, myelosuppression of rats induced with 5-FU/Leucovorin combinations of 30 mg/kg, administered according to Asao *et al.* at the maximum tolerated dose, did not affect the

level of and time to achieve anti-rat G17:DT antibody titers following immunization with the anti-G17(1-9)-DT immunogen.

In therapy studies using 5-FU/Leucovorin in combination with anti-G17(1-9)-DT, a potentiation of the 20 mg/kg and 12.5 mg/kg dosages was achieved. The 20 mg/kg dose was as effective as the maximum tolerated dose when combined with anti-G17(1-9)-DT, and the 12.5 mg/kg dose showed a trend to a greater therapeutic effect. The reason for the latter trend is not known but it may be due to the cytotoxic dose affecting the immune system to a lesser degree than higher dose levels, which may aid in the general inflammatory response against the tumor. 5-FU/Leucovorin given in continuous cycles would appear to exert an 'all or nothing' effect on tumor growth as lowering the dose to 1 mg/kg was found to exert no inhibition of tumor growth. The therapeutic effect may be titrated out more gradually by reducing the number of toxic cycles (Watson, personal communication). Therefore, in the combinations according to the present invention, lower than usual doses of 5-FU/Leucovorin can be administered, thus reducing the side effects of the drugs, while, at the same time, effective killing of tumor cells can be achieved using the present combination, since the immune system is only minimally affected. Thus, the growth inhibitory effect of anti-G17(1-9)-DT immunization is enhanced. These characteristics of the combination therapy are unexpected and surprising in view of the myelosuppressive effects of the chemotherapeutic agents by themselves.

Furthermore, by the absence of deleterious effects on the host, anti-G17(1-9)-DT immunization is likely to be a long-term treatment as shown by the length of time that measurable antibody levels were present in rats receiving a single immunization. The first immunization was shown to be 80% effective, in terms of anti-gastrin antibody induction, and 100% effective after the second immunization with an immediate rise in antibody levels. Although potentiation of chemotherapy may be achieved by a single anti-G17(1-9)-DT injection, in most hosts the absence of side effects, characteristic of anti-G17(1-9)-DT immunization, and the host response rate following boosts, indicate a multi-injection regime may be desirable. Despite the length of time that anti-rat-G17 antibodies remained in the circulation there appeared to be no long term deleterious effects on the GI tract, as determined by a simple histological assessment. Additionally, the crypt cell proliferation index of mucosal cells in the colon revealed no significant effect on their growth.

Example 6

Treatment of human colon cancer patients with a combination therapy of 5-FU/Leucovorin and anti-G17 (1-9)-DT.

Anti-G17(1-9)-DT immunization alone has previously been shown to be a valuable and safe therapeutic option in the treatment of gastrin-dependent cancer. The present combinations of anti-G17 immunogens with 5-FU/Leucovorin enhance the effectiveness of cancer treatment, in particular colon cancer treatment, and the possible reduction in the dosage of the chemotherapeutic agent required in the combination should reduce the deleterious cytotoxic side effects of any of the chemotherapeutic agents now in use. The present combinations of an immunogen with chemotherapeutic agents may also be useful as a second-line therapy in patients who do not respond to chemotherapy alone.

Human colorectal tumor or colon cancer patients are treated with a combination of chemotherapy and immunotherapy.

Specifically, for patients with gastrin responsive colorectal tumors or colon cancer can be treated with concomitant administration of 5-FU/Leucovorin and an anti-G17 immunogen composition or anti-G17 antibodies.

In particular, the preferred immunotherapy provides an immunogenic composition comprising an aminoterminal G17 (1-9) peptide: DT conjugate in a pharmaceutically acceptable carrier which may include an adjuvant to further stimulate the immune response.

The preferred immunotherapeutic regimen can start before, during or after the chemotherapy course depending on clinical considerations. For example, in a patient with a large tumor burden it may be advantageous to start with several cycles of chemotherapy to reduce the tumor bulk and then start with immunotherapy.

Alternatively, in a patient with a small tumor burden or after curative surgery, immunotherapy can be started before or during chemotherapy.

The active immunization dose can range between 300 µg up to 1200 µg of the anti-G17 immunogen, depending on the immune status of the patient (or the capacity of an immune response). The injection intervals can be on days 1, 7 and 14, or days 1, 14 and 21, or days 1, 14, then 28 and 56. All the schedules can result in similar antibody titers. The accelerated schedules of immunization provide the possibility of earlier onset of immune response.

The preferred method of the anti-gastrin therapy provides that a booster is administered every 6 months after the initial immunization period, regardless of which protocol is used.

Yet another preferred method for the effective neutralization of G17, Gly G17 and G17 NH₂ provides passive immunization with anti-G17 antibodies, preferably in purified form. More specifically, the inoculation of 10-1000 µg anti-G17(1-9) antibodies is administered before, during and/or after the chemotherapy cycles for the control of gastrin activities. The
5 passive immunization can be administered daily, weekly or biweekly. Other protocols can be followed depending on the effectiveness of the treatment.

A further combination of treatment provides for an initial passive immunization before and/or during the first cycle of chemotherapy followed by active immunization as described above.

10 Many chemotherapy regimens are in use. These art recognized regimens, although not described herein, are not excluded from the combination treatment according to this invention. One preferred chemotherapy regimen provides for 5-FU i.v. bolus of 425 mg/m² with i.v. infusion of Leucovorin (folic acid, FA, 20 mg/m²) for 1-5 days per period up to 4 weeks.

Another preferred regimen provides for 200 mg/m² FA over a period of 2h,
15 followed by 5-FU i.v. boles of 400 mg/m² + 5-FU of 600 mg/m² over 22 hours 1 or 2 days in a 2-week period.

Yet another preferred regimen provides for continuous infusion of 5-FU at 250-300 mg/m² day continuous i.v. for 4-6 weeks, followed by 2 weeks rest.

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SEQUENCE LISTING

<110> APHTON CORPORATION

<120> Combination Therapy for the Treatment of Tumors

<130> 1102865-0034

<140> US

<141> 1999-05-14

<150> US 60/085,687

<151> 1998-05-15

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<213> Artificial Sequence

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<223> Description of Artificial Sequence:Synthetic
peptide

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What we claim is:

1. A method for treating a tumor in a patient, comprising immunologically neutralizing a tumor-growth factor and administering to the patient an effective amount of one or more chemotherapeutic agents.
- 5 2. The method of claim 1, wherein the tumor is a gastrin-dependent tumor.
3. The method of claim 1, wherein the tumor-growth factor is gastrin.
4. A method for treating a gastrin-dependent tumor with a combination therapy, comprising administering to a mammal in need of said treatment a therapeutically effective amount of an anti-gastrin 17 immunogen, in combination with one ore more chemotherapeutic agents.
- 10 5. The method of claim 4, wherein the antigastrin-G17 immunogen is conjugated to Diphtheria toxoid.
6. The method of claim 4, wherein the antigastrin-G17 immunogen further comprises a spacer peptide.
7. The method of claim 4, wherein the antigastrin-G17 immunogen comprises a peptide consisting of amino acid sequence pGlu-Gly-Pro-Trp-Leu-Glu-Glu-Glu-Glu (SEQ ID NO.: 1 in the Sequence Listing).
- 15 8. The method of claim 4, wherein the chemotherapeutic agents are 5-fluorouracil and leucovorin.
9. The method of claim 4, wherein the antigastrin-G17 immunogen is administered prior to administering 5-fluorouracil and leucovorin chemotherapy.
10. The method of claim 4 or 9, wherein the chemotherapeutic agents are administered in several cycles during the therapy.
- 20 11. The method of claim 1 or 4, further comprising administering one or more booster immunizations.

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 A61K31/505 A61K39/395 A61K31/385 //A61K39/39, A31K39:505

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
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☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"Z" document member of the same patent family

Date of the actual completion of the international search

18 November 1999

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25/11/1999

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
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| Y | WATSON S A ET AL: "ANTI-GASTRIN ANTIBODIES RAISED BY GASTRIMMUNE INHIBIT GROWTH OF THE HUMAN COLORECTAL TUMOUR AP5" INTERNATIONAL JOURNAL OF CANCER, US, NEW YORK, NY, vol. 61, no. 2, page 233-240 XP000654518 ISSN: 0020-7136 the whole document ---- | 1-11 |
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Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 1-11 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

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